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ORIGINAL ARTICLE

Dicloxacillin-warfarin drug–drug interaction—A register-based study and in vitro investigations in 3D spheroid primary human hepatocytes

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Aims: Dicloxacillin is used to treat staphylococcal infections and we have previously shown that dicloxacillin is an inducer of cytochrome P450 enzymes (CYPs). Here, we employed a translational approach to investigate the effect of a treatment with dicloxacillin on warfarin efficacy in Danish registries. Furthermore, we assessed dicloxacillin as an inducer of CYPs in vitro.

Methods: We conducted a register-based study and analysed international normalized ratio (INR) levels in chronic warfarin users before and after short- and long-term use of dicloxacillin ($n = 1023$) and flucloxacillin ($n = 123$). Induction of CYPs were investigated in a novel liver model of 3D spheroid primary human hepatocytes at the level of mRNA, and protein and enzyme activity.

Results: Short- and long-term dicloxacillin treatments decreased INR levels by −0.65 (95% confidence interval [CI]: −0.57 to −0.74) and −0.76 (95% CI: −0.50 to −1.02), respectively. More than 90% of individuals experienced subtherapeutic INR levels (below 2) after long-term dicloxacillin treatment. Flucloxacillin decreased INR levels by −0.37 (95% CI: −0.14 to −0.60). In 3D spheroid primary human hepatocytes, the maximal induction of CYP3A4 mRNA, protein and enzyme activity by dicloxacillin were 4.9-, 2.9- and 2.4-fold, respectively. Dicloxacillin also induced *CYP2C9* mRNA by 1.7-fold.

Conclusion: Dicloxacillin induces CYPs and reduces the clinical efficacy of warfarin in patients. This effect is substantially exacerbated during long-term treatment with dicloxacillin. The in vitro results corroborated this drug–drug interaction and correlated to the clinical findings. Caution is warranted for warfarin patients that initiate dicloxacillin or flucloxacillin, especially for a long-term treatment of endocarditis.

KEYWORDS
anticoagulant, CYP, drug–drug interactions, induction, INR monitoring, primary human hepatocytes, warfarin

1 | INTRODUCTION

Drug–drug interactions (DDIs) cause challenges in drug therapy and can lead to adverse effects or decreased efficacy of drugs. **Warfarin**, a widely used anticoagulant, is a well-known victim drug for numerous DDIs because of its narrow therapeutic index. In a previous registry-based study, we found that initiation of **dicloxacillin** leads to decreased international normalized ratio (INR) levels in warfarin-treated individuals ($n = 236$).[1] Similarly, a Swedish register-based study found decreased INR levels in warfarin-treated individuals after 10 days treatment with **flucloxacillin** ($n = 5848$), while the effect was more pronounced after 30 days treatment ($n = 201$).[2] Furthermore, we found that both antibiotics were associated with increased risk of ischaemic stroke or systemic embolism in patients under warfarin therapy and diagnosed with atrial fibrillation or heart valve replacement.[3] In line with the studies that analysed INR levels, the decrease in efficacy of warfarin (risk of ischaemic stroke or systemic embolism) was more pronounced for dicloxacillin than flucloxacillin.

Dicloxacillin is an antibacterial drug that inhibits bacterial cell wall synthesis and belongs to the group of β-lactamase resistant penicillins along with cloxacillin, flucloxacillin, methicillin, nafcillin and oxacillin.[4,5] Dicloxacillin is primarily eliminated by renal excretion with only a minor contribution from drug metabolism.[4–6] A typical dicloxacillin treatment course lasts for 7–10 days; however, the treatment of staphylococcal endocarditis requires administration of dicloxacillin for several weeks. Our previous clinical DDI study in healthy volunteers found that 10 days' treatment with dicloxacillin decreased the exposure of buccal midazolam and oral tolbutamide, probes of cytochrome P450 enzymes (CYPs) **3A4** and **2C9**, by 1.9- and 1.3-fold, respectively.[7] Both of these CYPs are the main enzymes responsible for warfarin metabolism.[8–10]

The pharmacokinetic DDI between warfarin and dicloxacillin has never been evaluated in a clinical study. Nevertheless, a case report found that initiation of dicloxacillin decreased plasma concentrations of warfarin isomers by 20–25% after 5 days treatment in a patient under warfarin therapy.[11] In contrast, 7 days treatment with amoxicillin–clavulanic acid combination did not affect plasma concentrations of warfarin isomers or INR levels in a randomized controlled trial.[12] This is in line with an in vitro study in primary human hepatocytes (PHHs) that found no effect of amoxicillin or phenoxymethylpenicillin on the expression of CYP3A4.[13] Infections decrease the activity of CYPs,[14] while infection may also increase INR levels and predispose patients to bleeding events.[15–17] Thus, the DDI between dicloxacillin and warfarin is not attributed to a general effect of antibiotics but rather a specific pharmacokinetic DDI.

Quantitative prediction of clinical DDIs based on in vitro induction data is challenging.[18–20] Particularly, induction responses in the standard 2D culture system of PHHs may vary highly both within and between donors.[20] Moreover, it has become obvious that mRNA as an endpoint for in vitro–in vivo extrapolation (IVIVE) may be suboptimal as mRNA does not always correlate to protein or enzyme activity in vitro.[21–23] Our previous in vitro study in 2D cultured PHHs found that dicloxacillin increases *CYP3A4* mRNA by 30-fold.[7] Clearly, such a magnitude of induction does not translate to the clinical magnitude of dicloxacillin-mediated CYP3A4 interactions. Recently, 3D spheroid PHHs were shown to better reproduce clinically relevant induction of CYP3A4.[24,25]

In this study, we assessed if initiation of dicloxacillin use is associated with altered warfarin efficacy in a large Danish cohort of chronic warfarin users during both short- and long-term dicloxacillin treatment. Additionally, we investigated in vitro induction of CYPs by dicloxacillin in a novel liver model of 3D spheroid cultured PHHs. We hypothesized that 3D spheroid cultured PHHs provide a better estimate of DDIs than the traditional 2D culture of PHHs.

What is already known about this subject

- Dicloxacillin and flucloxacillin induce cytochrome P450 enzymes (CYPs) in humans.
- A previous analysis revealed that dicloxacillin treatment decreases international normalized ratio (INR) levels in warfarin-treated individuals ($n = 236$). This study did not assess differences between short- and long-term treatment.
- A previous study in 2D cultured primary human hepatocytes (PHHs) overestimated dicloxacillin mediated CYP3A4 induction.

What this study adds

- Dicloxacillin ($n = 1023$) and flucloxacillin ($n = 123$) treatment strongly affects INR levels in warfarin-treated individuals.
- Long-term treatment with dicloxacillin in warfarin-treated patients causes >90% of patients to experience subtherapeutic INR levels.
- 3D spheroid PHHs provide more accurate estimates of induction by dicloxacillin than 2D cultured PHHs.

2 | METHODS

2.1 | Registry-based study

We conducted a self-controlled cohort study using the unique Danish registers. Within the Danish National Prescription Registry,[26] we identified warfarin users with any new use of dicloxacillin (exposure). Inclusion criteria were age ≥18 years, a prescription for warfarin within 180 days of prior the exposure to dicloxacillin, and measurement of INR before (within 8 weeks) and after (within 12 weeks) of the exposure. INR data were obtained from the Copenhagen Primary

Care Laboratory (CopLab) database,[27] which covered approximately 1.3 million individuals. The accurate linkage of data was ensured using the Danish unique personal identifier.[28] The data for register-based study were collected between 2000 and 2015.

The outcome of interest was a change in INR levels following the exposure of dicloxacillin. Our primary analysis was to compare the first INR measurement within 1–3 weeks after the exposure with the last INR measurement within 3–5 weeks before the exposure. Furthermore, we assessed the proportion of individuals with 1 INR measurement below the therapeutic limit (INR < 2) 3–5 weeks before the exposure compared with 1–3 weeks after the exposure. In a subgroup analysis, we assessed the impact of short-term and long-term dicloxacillin treatment. Individuals with a prescription for ≤30 g dicloxacillin were considered to receive a short-term dicloxacillin treatment, corresponding to 10 days of treatment. Individuals with a prescription for >30 g were considered to receive dicloxacillin therapy of longer duration. Lastly, we applied the same analysis in groups consisting of individuals with exposure to flucloxacillin (combined short- and long-term prescriptions), amoxicillin (control group) and phenoxymethylpenicillin (control group).

2.2 | In vitro studies

2.2.1 | Materials

Primary human hepatocytes were acquired from Thermo Fisher Scientific (Waltham, MA, USA) or from BioIVT (Baltimore, MD, USA). The hepatocyte lots (HU8345-A, HU8339-A and BGF) were prequalified for spheroid formation by the suppliers (see Table S1 for donor information). All cell culture reagents were from Thermo Fisher Scientific. Dicloxacillin and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Qiazol reagent for RNA extraction was from Qiagen (Germantown, MD, USA). Glycogen (RNA grade), cDNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor), qPCR reagents (TaqMan assay) and trypsin for protein digestion (mass spectrometry [MS]-grade Pierce Trypsin Protease) were from Thermo Fisher Scientific. Enzyme activity probes, analytical standards for metabolites and internal standards were from Chiron AS (Trondheim, Norway), Toronto Research Chemicals (Toronto, ON, Canada) or Sigma-Aldrich.

2.2.2 | 3D spheroid culture of PHHs

3D spheroid culture of PHHs has been previously reported.[29] On day 0, 1500 hepatocytes were transferred to each well of an ultra-low attachment 96-well plates, the plates were centrifuged for 2 min at 200*g* and transferred to a cell culture incubator (+37°C and 5% CO_2) for 5 days. The total volume of cell culture medium was 100 µL per well and contained 5% foetal bovine serum, 1 µM dexamethasone, 5 µg/mL human recombinant insulin, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (GlutaMAX supplement) and 15 mM HEPES in Williams' E medium. Spheroids formed within 5 days of culture and on days 5–7 70% of medium for each well was changed to a maintenance medium containing 0.1 µM dexamethasone, 10 µg/mL human recombinant insulin, 5.5 µg/mL transferrin, 6.7 ng/mL selenium, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (GlutaMAX supplement) in William's E medium. In the case of lots HU8345-A and HU8339-A (Thermo Fisher Scientific), the maintenance medium also contained 5.35 µg/mL linoleic acid, 1.25 mg/mL bovine serum albumin and 15 mM HEPES. However, the manufacturer (Thermo Fisher Scientific) does not anymore recommend using these supplements (personal communication).

2.2.3 | Dicloxacillin treatments

Dicloxacillin treatments were conducted for 4 days between culture days 8 and 12. On day 10, 70% of medium, containing dicloxacillin or vehicle, was changed for the treatments. The final concentrations of dicloxacillin were 0.15, 1, 5, 20, 45, 80 and 250 µM, while the vehicle control was 0.1% DMSO. We have shown positive induction response in 3D spheroid PHHs by different probe inducers, such as rifampicin, phenobarbital and omeprazole.[25]

2.2.4 | mRNA expression analysis

For RNA extraction, pools of spheroids were collected and media was removed before lysis with 500 µL of Qiazol reagent and storage at −80°C. RNA was extracted with chloroform-phenol method according to the manufacturer's protocol of Qiazol. RNA was coprecipitated with 15 µg of glycogen. cDNA was synthesized from RNA (400–600 ng) and subsequently employed for real-time PCR with TaqMan Universal Master Mix II and TaqMan assays. The target specific TaqMan assays were Hs02758991_g1 (*GAPDH*), Hs00167927_m1 (*CYP1A2*), Hs04183483_g1 (*CYP2B6*), Hs00946140_g1 (*CYP2C8*), Hs04260376_m1 (*CYP2C9*), Hs00426380_m1 (*CYP2C19*), Hs00164385_m1 (*CYP2D6*) and Hs00604506_m1 (*CYP3A4*). Expression of *GAPDH* was used for sample normalization and the resulting ΔCt values for every sample were transformed by 2^{-Ct}. Finally, the relative expression of each dicloxacillin concentration was calculated by dividing expression values by the mean value of 0.1% DMSO group separately for each donor and target gene.

2.2.5 | Protein expression analysis

Relative protein expression after dicloxacillin treatments was determined for donors HU8345-A and HU8339-A. Spheroids were washed once with phosphate-buffered saline solution after complete removal of wash solution and storage of samples at −80°C. Spheroids were denatured in 42 mM ammonium bicarbonate buffer containing 1.14 mM dithiothreitol and 9 mM iodoacetamide for 15 min at 90°C.[22] Proteins were digested with trypsin for 16 h at 37°C. Digestions were stopped by addition of phenylmethylsulfonyl fluoride to a

final concentration of 1 mM and a final sample volume of 70 µL, and 25 µL of the sample was used for the protein quantification. The targeted liquid chromatography (LC)–MS method and triple X proteomics antibody precipitation of peptides have been previously reported.[30]

2.2.6 | Enzyme activity assays

Enzyme activity of CYPs was determined for donors HU8345-A and BGF. For the enzyme activity assay, the Basel cocktail (modified from Berger *et al.*[31]) was employed at concentrations of 160 µM caffeine (CYP1A2), 20 µM efavirenz (CYP2B6), 30 µM losartan (CYP2C9), 30 µM omeprazole (CYP2C19), 40 µM metoprolol (CYP2D6) and 10 µM midazolam (CYP3A4). After 4-day treatments with the different dicloxacillin concentrations and vehicle, spheroids were washed 3 times with the maintenance medium. The Basel cocktail was applied to each well in a final volume of 100 µL. Spheroids were incubated either for 0.5 h (for the analysis of 5-hydroxyomeprazole formation) or 8 h (for the analysis of other metabolites) before the medium and spheroid were collected from each well and stored at −80°C. The enzyme activity assay was optimized elsewhere.[25] Samples were subjected to LC–MS analysis as described in Materials S1.

2.3 | Data and statistical analysis

Register-study data are described with median and interquartile range (IQR) or mean and 95% confidence intervals (95% CI). Changes in INR (after *vs.* before) was tested by paired *t*-test and changes in the proportion of individuals with INR < 2 (after *vs.* before) was tested with Fisher's exact test. Statistical significance is only stated where $P \le 0.05$. Statistical analyses were done in Stata (Stata Corporation, College Station, Texas, USA) and R programming language (version 4.2.2, R Core Team 2022).

In vitro experiments included 2 independent (protein expression and enzyme activity) or 3 independent (mRNA expression) experiments each with different donor of PHHs (Table S1). For protein and mRNA expression, each dicloxacillin concentration or vehicle group included 48 spheroids that were divided in triplicate pools for analyses. In enzyme activity assays, each dicloxacillin concentration or vehicle group included 2 or 3 wells (spheroid + media). Each individual experiment was normalized to the mean value of vehicle group and data are presented as mean values relative to the vehicle group.

Concentration-dependent induction data (7 concentrations of dicloxacillin and vehicle) of mRNA, protein or enzyme activity levels from 3D PHH studies were fitted to the logistic 3-parameter equation[32]:

$$y = 1 + \frac{E_{max} - 1}{1 + 10^{\log(EC_{50}) - \log(x)}} \quad (1)$$

where y is the fold-increase of mRNA, protein or enzyme activity at the inducer concentration of x, E_{max} is the maximum induction response and EC_{50} is the concentration producing half of the maximum induction.

Data were fitted with nonlinear least squares (nls) function in R programming language (version 4.2.2, R Core Team 2022). The resulting fitted parameters E_{max} and EC_{50} are presented as best fits and 95% CIs of the fitting.

2.4 | IVIVE

To predict the magnitude of clinical DDI from in vitro parameters, an estimation of maximum hepatic inlet concentration (I_h) of dicloxacillin (Equation 2) and combining I_h with the vitro parameters (Equation 3) are needed.[33,34] The mechanistic static model (Equation 3) and estimation of I_h (Equation 2) are recommended by the US Food and Drug Administration for the estimation of clinical DDI based on the in vitro DDI parameters and the plasma concentration of perpetrator drug.[35]

$$I_h = f_{u,p} \times \left(C_{max} + \frac{F_a \times F_g \times k_a \times Dose}{Q_h \times R_B} \right) \quad (2)$$

where $f_{u,p}$ is the unbound fraction of drug in plasma, C_{max} is the maximum concentration of drug in plasma, F_a is the fraction absorbed after oral administration, F_g is the fraction available after intestinal metabolism, k_a is the first order absorption rate constant, Q_h is the hepatic blood flow and R_B is the blood-to-plasma concentration ratio of drug.

To estimate I_h for dicloxacillin in humans after 1-g dosing (Dose), the parameters were set as following: $f_{u,p}$ as 0.03 (reported[36]), C_{max} as 64 µM (reported[36]), F_a as 1 (no human data available), F_g as 1 (no human data available), k_a 0.1 min^{-1} (maximum estimated[37]), Q_h as 1610 mL/min[37] and R_B as 0.63 (reported for flucloxacillin,[38] a chemically highly similar compound). The resulting I_h, 8.2 µM, was used in Equation (3) along with the derived in vitro parameters (Equation 1) to calculate the area under the plasma concentration-time curve ratio (AUCR) values for each CYP enzyme.

For the prediction of relative change of drug exposure (AUCR, Equation 3), only hepatic induction is included since in vitro parameters here were available only from hepatocytes. The model also assumes that the victim drug is solely metabolized by the induced enzyme meaning that the fraction metabolized is 1.

$$AUCR = \frac{1}{1 + \frac{(E_{max} - 1) \times I_h}{EC_{50} + I_h}} \quad (3)$$

2.5 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[39]

3 | RESULTS

3.1 | Effect of dicloxacillin on warfarin efficacy in a registry-study

We identified 1023 individuals initiating dicloxacillin while being under warfarin therapy. The median age was 78 years (IQR: 69–84), and 54% were male. Initiation of short- or long-term dicloxacillin in warfarin users decreased INR levels within 1–3 weeks of exposure by a mean of −0.65 (95% CI: −0.57; −0.74, $n = 566$) and −0.76 (95% CI: −0.50; −1.02, $n = 105$), respectively (Figure 1). The effect of dicloxacillin on INR levels was largest after 2 weeks of initiation (Figure 1) and >90% of all individuals in the long-term exposure group experienced subtherapeutic INR (<2; Figure 1B). Long-term exposure of dicloxacillin caused subtherapeutic INR levels for up to 6 weeks in half of the study population (Figure 1B), while in the short-term dicloxacillin exposure group, INR levels were below 2 for 3 weeks in half of the study population (Figure 1A). In the overall analysis, initiation of dicloxacillin (combined short- and long-term treatments) caused a decrease in the mean INR levels from 2.50 to 1.84 within 1–3 weeks of exposure, with a mean difference of −0.67 (95% CI: −0.59; −0.75, $P < 0.001$, $n = 671$). A total of 70% of the individuals experienced INR levels below the therapeutic range (INR < 2) within 1–3 weeks after dicloxacillin, compared with 27% in a period of 3–5 weeks preceding dicloxacillin ($P < 0.01$; Figure 2A).

The flucloxacillin cohort ($n = 123$) consisted of 59% male with a median age of 83 years (IQR 74–86). Similar to dicloxacillin, flucloxacillin treatment, caused a reduction in INR levels with a mean difference of −0.37 (95% CI: −0.14; −0.60, $P < 0.01$, $n = 75$). Low INR levels (INR < 2) were observed among 42% of individuals 1–3 weeks after flucloxacillin treatment compared with 21% in a period of 3–5 weeks preceding flucloxacillin treatment ($P = 0.05$; Figure 2B).

The amoxicillin cohort ($n = 669$) consisted of 58% males with a median age of 75 years (IQR 66–82) while the phenoxymethylpenicillin cohort ($n = 1458$) consisted of 54% males with a median age of 77 years (IQR 69–83). Amoxicillin caused a mean increase in INR levels of 0.21 (95% CI: 0.09; 0.32, $P < 0.001$, $n = 410$; Figure 2C), and phenoxymethylpenicillin caused a mean increase in INR levels of 0.07 (95% CI: 0.00; 0.14, $P < 0.05$, $n = 845$; Figure 2D).

3.2 | Induction of CYPs in 3D spheroid PHHs by dicloxacillin

Induction of CYP enzymes in vitro was investigated in 3D spheroid PHHs after 4 days of dicloxacillin exposure. CYP3A4 was induced at the mRNA, protein and enzyme activity levels resulting in E_{max} of 2.4–4.9 and EC_{50} of 7.3–14 μM (Figure 3G and Table 1). CYP2C9 was weakly induced by dicloxacillin and the maximum induction of *CYP2C9* mRNA and protein were 1.7- and 1.3-fold (Figure 3D and Table 1), respectively. The EC_{50} values were similar between mRNA and protein induction for CYP2C9 (Table 1). In the case of CYP2B6 and CYP2C8, the induction of mRNA levels was 2–3-fold higher than the induction of protein levels (Figure 3B,C, Table 1).

Dicloxacillin did not induce CYP1A2 or CYP2D6 at the mRNA or protein levels (Figure 3A,F), while the enzyme activity of CYP2D6 (alpha-hydroxylation of metoprolol) was induced (Figure 3F). CYP2C19 was noninducible in 2 donors both at the mRNA and

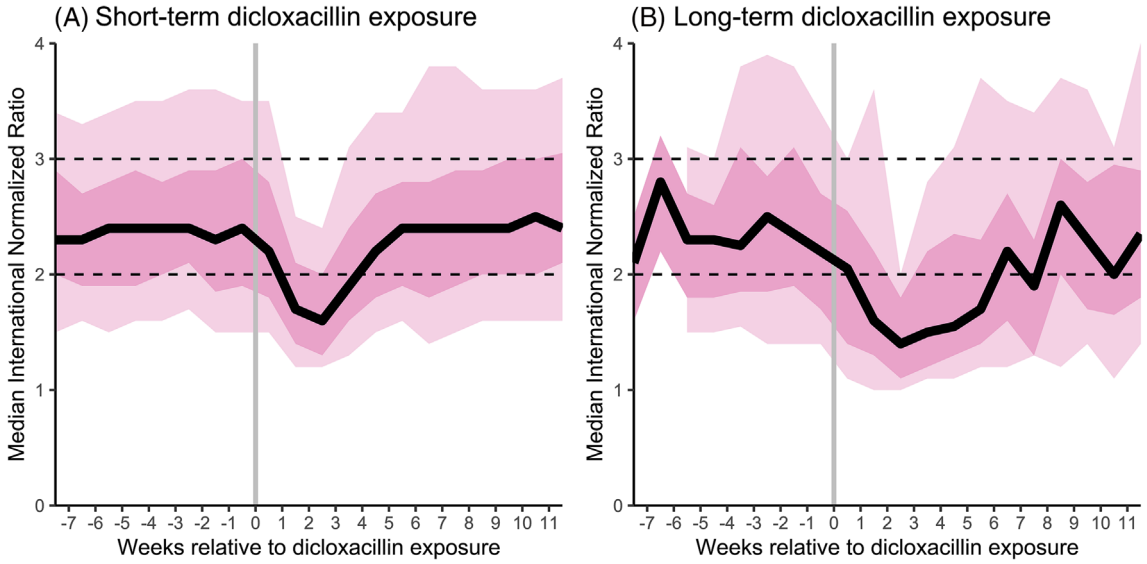

FIGURE 1 Effect of short-term dicloxacillin exposure (a prescription for 10 days or less, A) and long-term dicloxacillin exposure (a prescription for more than 10 days, B) on international normalized ratio levels among chronic warfarin users. Data are presented as median (thick line), 25th to 75th percentile (dark shade) and 10th to 90th percentile (pale shade). The grey vertical line indicates the time of dicloxacillin prescription. Dotted horizontal lines present the therapeutic range of warfarin (international normalized ratio 2–3). $n = 1063$.

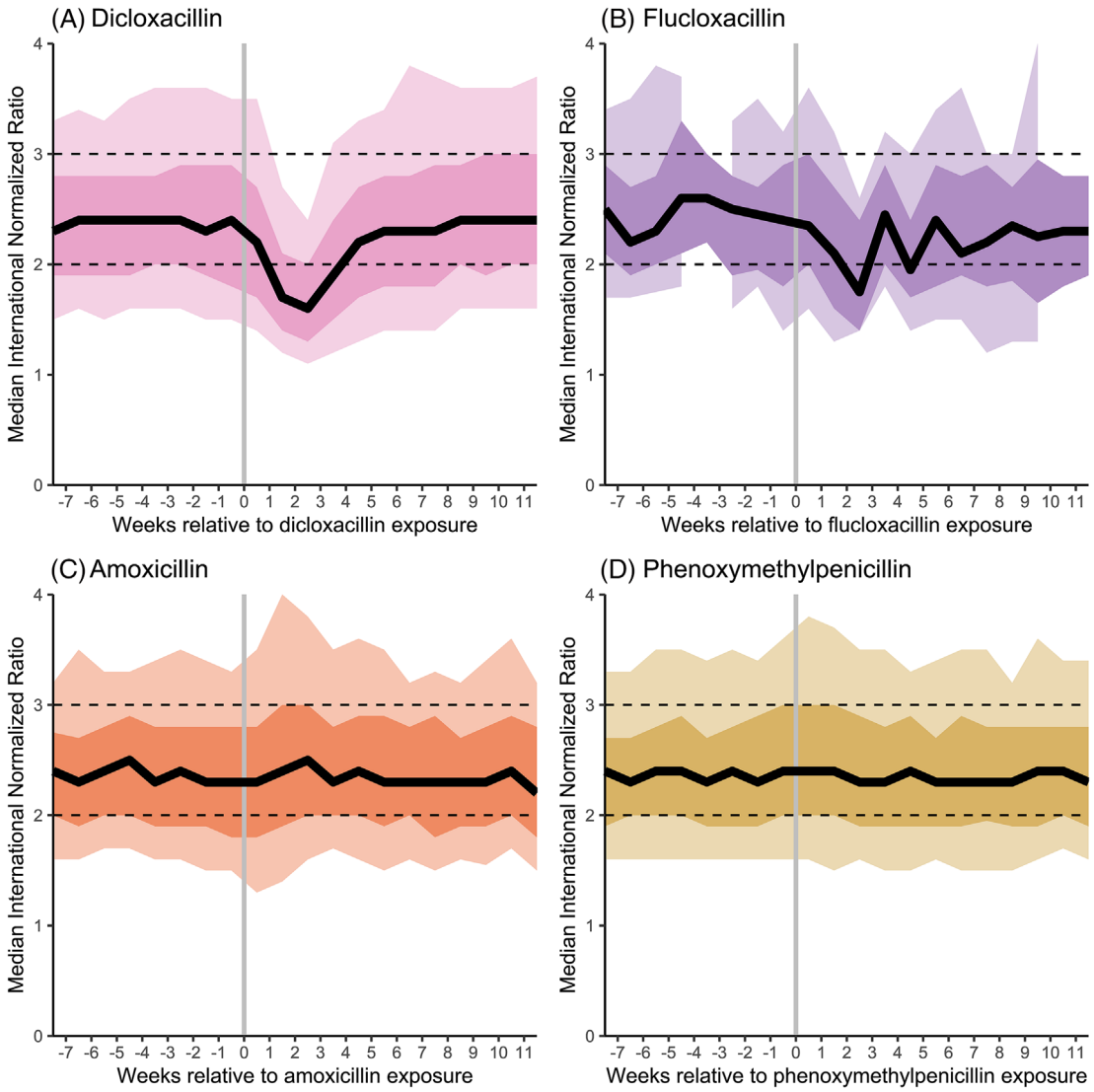

FIGURE 2 Effect of initiation of dicloxacillin (A, $n = 1063$), flucloxacillin (B, $n = 123$), amoxicillin (C, $n = 669$) and phenoxymethylpenicillin (D, $n = 1458$) on international normalized ratio levels among chronic warfarin users. Data are presented as median (thick line), 25th to 75th percentile (dark shade) and 10th to 90th percentile (pale shade). The grey vertical line indicates the time of antibiotic prescription. Dotted horizontal lines present the therapeutic range of warfarin (international normalized ratio 2–3).

protein levels (Figure 3E). However, in both investigated donors, the activity of CYP2C19 was inducible (Figure 3E). Limited or no induction of other CYP enzymes was found (Figure S1).

3.3 | Prediction of in vivo effect of dicloxacillin on CYPs

The mechanistic static model was employed to predict the magnitude of clinical DDIs mediated by dicloxacillin-mediated induction of CYPs. We calculated the maximum, unbound hepatic concentration of dicloxacillin in humans after oral administration (see Section 2.3) and incorporated in vitro parameters of induction from 3D spheroid PHHs for different CYPs (Table 1) in the mechanistic model. The relative change in exposure of a victim drug (AUCR) that is solely metabolized by a specific CYP enzyme was predicted (Table 1).

Dicloxacillin was predicted to have the highest effect on CYP3A4—AUCRs of 0.40, 0.57 and 0.59 when mRNA, enzyme activity and protein were used as a source of in vitro parameters, respectively (Table 1). The predicted AUCRs for CYP2C9 were 0.75 and 0.92 based on mRNA and protein induction, respectively (Table 1).

To further understand the performance of 3D spheroid PHHs in comparison to the traditional 2D monolayer culture of PHHs, we performed similar prediction on our previously published data from 2D cultured PHHs[7] (Table S2). The AUCR values for CYP3A4 were 2-fold lower resulting in higher prediction of clinical DDI magnitude for 2D monolayer PHHs compared with 3D spheroid PHHs (Tables 1 and S2). In the case of *CYP2C9* mRNA induction, both 2D monolayer

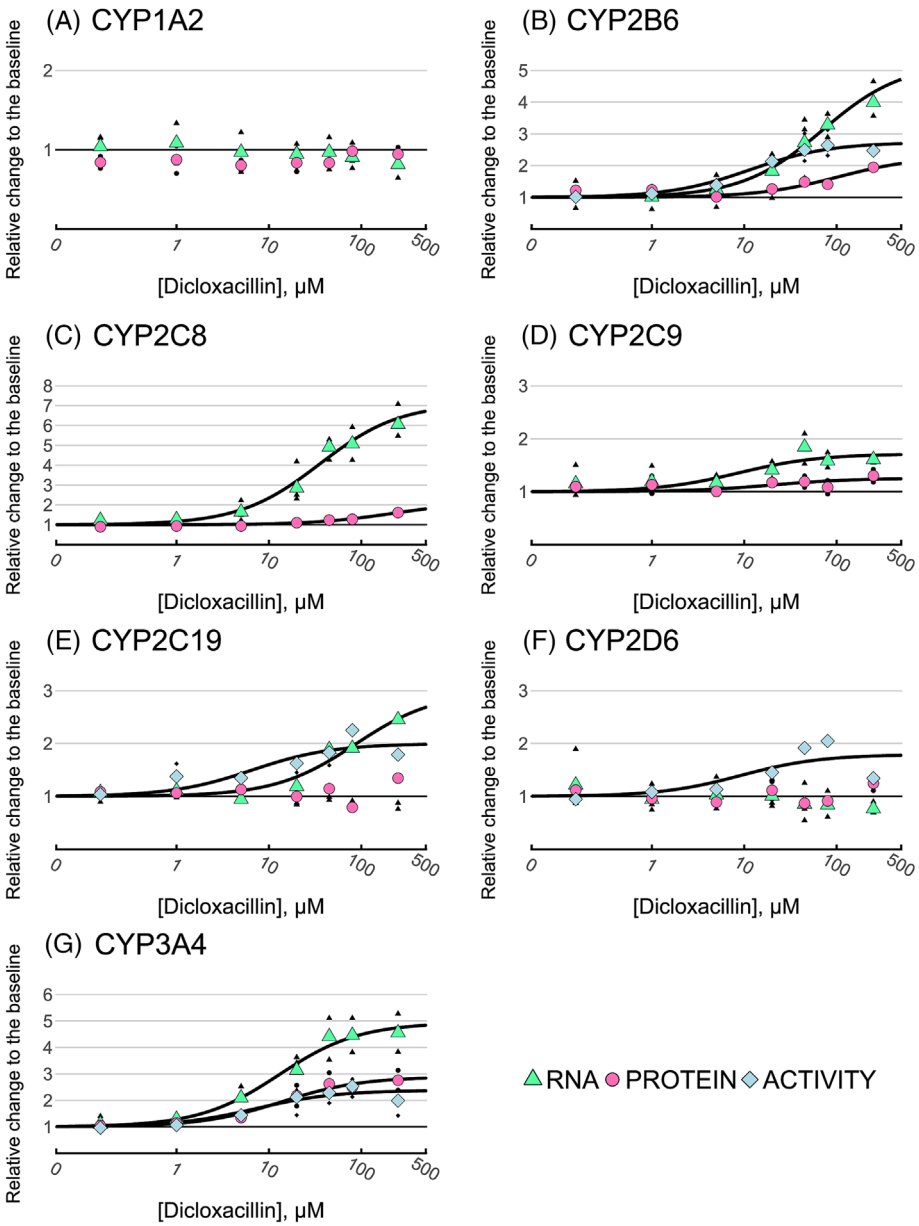

FIGURE 3 In vitro induction of cytochrome P450 enzymes (CYPs) by dicloxacillin in 3D spheroid primary human hepatocytes. Different concentrations (0.15–250 μM) of dicloxacillin were applied to spheroids that were treated for 4 days. Data were normalized to the mean value of vehicle (0.1% dimethyl sulfoxide) group for each donor and are presented as mean values of all donors. Two donors (HU8339-A and BGF) were excluded from the mean calculations of CYP2C19 mRNA induction since no induction was found. The lines present the fittings of data to the logistic 3-parameter equation and the fitted parameters are reported in Table 1. $n = 3$ donors (mRNA) or 2 donors (protein and enzyme activity) each presented as mean values of triplicate pools of 16 spheroids and shown as black-filled shapes.

and 3D spheroid PHHs resulted in similar AUCR predictions: 0.80 for 2D monolayer PHHs (Table S2) and 0.75 for 3D spheroid PHHs (Table 1).

4 | DISCUSSION

We show for the first time that long-term treatment with dicloxacillin leads to dramatically reduced efficacy of warfarin. Furthermore, we confirmed our previous finding that short-term treatment with dicloxacillin causes a pronounced reduction in INR levels among individuals with chronic warfarin use. A similar, but less dramatic, pattern was observed for flucloxacillin. This is not caused by the underlying infection itself, as this effect was not observed among individuals treated with amoxicillin and phenoxymethylpenicillin, but rather by induction of CYP enzymes by dicloxacillin and flucloxacillin. We utilized 3D spheroid PHHs, a novel and advanced in vitro model of human liver, to show induction of CYPs by dicloxacillin, yielding results supporting the interpretation of CYP induction as an underlaying reason for the effect of dicloxacillin on warfarin efficacy. Finally, we employed IVIVE with a static modelling to highlight that 3D spheroid PHHs predict the clinical impact of CYP induction by dicloxacillin better than 2D PHHs.

We found that long-term dicloxacillin exposure (>10 days) resulted in prolonged subtherapeutic INR levels in chronic warfarin users (Figure 1B). More than 90% of individuals experienced subtherapeutic INR levels (<2) after 2 weeks' dicloxacillin treatment and that this effect persisted for half of the individuals for up to 6 weeks after dicloxacillin initiation (Figure 1B). Initiation of dicloxacillin, both short- and long-term, decreased mean INR levels from 2.50 to 1.84 in individuals taking warfarin ($n = 1023$) and 70% of them experienced

TABLE 1 The fitted parameters for dicloxacillin-mediated induction.

Enzyme	Protein[a]	Activity[b]	mRNA[c]	Hepatic AUCR based on protein induction	Hepatic AUCR based on enzyme activity induction	Hepatic AUCR based on mRNA induction
CYP2B6	E_{max} = 2.3 EC_{50} = 105 μM	E_{max} = 2.7 (2.5–3.0) EC_{50} = 11 μM (6.1–21)	E_{max} = 5.2 (4.6–6.1) EC_{50} = 71 μM (45–111)	0.91	0.58	0.70
CYP2C8	E_{max} = 2.2 EC_{50} = 225 μM	ND	E_{max} = 7.1 (6.6–7.8) EC_{50} = 35 μM (26–48)	0.96	NE	0.46
CYP2C9	E_{max} = 1.3 EC_{50} = 20 μM	ND	E_{max} = 1.7 (1.6–1.9) EC_{50} = 8.8 μM (3.1–20)	0.92	NE	0.75
CYP2C19	NI	E_{max} = 2 (1.8–2.3) EC_{50} = 6.6 μM (1.2–22)	E_{max} = 3.0 (2.6–3.4) EC_{50} = 87 μM (56–139)	NE	0.64	0.85
CYP2D6	NI	E_{max} = 1.8 EC_{50} = 9.2 μM	NI	NE	0.73	NE
CYP3A4	E_{max} = 2.9 (2.6–3.2) EC_{50} = 14 μM (7.0–26)	E_{max} = 2.4 (2.1–2.7) EC_{50} = 7.2 μM (2.9–16)	E_{max} = 4.9 (4.6–5.3) EC_{50} = 13 μM (9.1–18)	0.59	0.57	0.40

Note: E_{max} is presented as a relative induction to the vehicle (value of 1) and EC_{50} as a concentration of dicloxacillin (μM), both with 95% confidence intervals of the fitting in parentheses. For CYP2C19, donors 2 and 3 were not inducible (mRNA and protein) or not detectable in donor 1 (protein), and thus were excluded from the analysis. For the protein induction of CYP2B6, CYP2C8 and CYP2C9, and for the enzyme activity induction of CYP2D6, no 95% confidence intervals could be calculated. The data and fittings of models are presented in Figure 2.

Abbreviations: CYP, cytochrome P450; E_{max}, maximum induction; EC_{50}, concentration at half maximum induction; ND, not determined; NE, not evaluated; NI, no induction.

[a] n = 2 donors or 1 donor in the case of CYP2B6.

[b] n = 2 donors.

[c] n = 3 donors or 1 donor in the case of CYP2C19.

INR levels below 2 within 1–3 weeks after dicloxacillin prescription. These estimates are similar to our previous registry-based study ($n = 236$), in another Danish region, in which dicloxacillin caused a drop in INR levels (2.59 to 1.97) and 61% of the individuals experienced INR levels below 2.[1] Dicloxacillin increases the risk of ischaemic stroke and systemic embolism in atrial fibrillation or heart valve replacement patients under warfarin therapy.[3] Our findings indicate that this might be even more pronounced during long-term treatment with dicloxacillin but this needs confirmation in appropriately designed studies.

Flucloxacillin initiation lowered INR levels (Figure 2A,B) but the effect was weaker with a mean decrease of 0.37 compared with 0.67 for dicloxacillin. Flucloxacillin caused 42% of the individuals to experience subtherapeutic INR levels after 1–3 weeks compared with 21% before flucloxacillin. These findings are in line with a previous Swedish registry-based study in which the proportion of individuals with a subtherapeutic INR before and after flucloxacillin were 22 and 35% for short-term treatment and 35 and 65% for long-term treatment.[2] Flucloxacillin is a weaker inducer of CYP enzymes than dicloxacillin in vivo and in vitro,[7,25,40,41] which aligns well with our results here regarding the differences between these antibiotics. In contrast, amoxicillin or phenoxymethylpenicillin did not decrease INR levels after their initiation but caused a small increase (Figure 2C,D). Neither amoxicillin nor phenoxymethylpenicillin induce CYP3A4 in vitro.[13] Infections decrease CYP activity in vivo and thus may increase warfarin levels, which may explain the small increase in INR values following administration of antibiotics.[14]

IVIVE of DDIs caused by induction is typically adjusted with correction factors or by correlation methods, especially for CYP3A4.[33,34,42] Such methods are needed because of a pronounced variability in the in vitro estimation of CYP3A4 induction. This is likely not related to interindividual variability but rather to the weak performance of 2D culture format of PHHs.[20,24] Here, we predicted AUCR of 0.4 and 0.57 for CYP3A4 based on dicloxacillin-mediated induction of mRNA and enzyme activity (Table 1). When re-analysing our previously published data for dicloxacillin mediated CYP induction in 2D cultured PHHs,[7] we found that this in vitro model overpredicted the clinical DDI for CYP3A4 by about 2-fold (Table S2). In our previous clinical pharmacokinetic trial, 10 days treatment of dicloxacillin decreased the AUC of buccal midazolam to 0.54, which aligns with the predictions provided by data from 3D spheroid PHHs here based on protein (AUCR of 0.59) and enzyme activity (AUCR of 0.57; Table 1). In 3D spheroid PHHs, the baseline expression of CYP3A4 is higher and more stable,[24,25] which may lead to better estimates of in vivo induction allowing IVIVE without correction factors. The general applicability of this needs to be assessed in future research.

CYP2C9 is mainly responsible for the metabolism of the more active S-warfarin enantiomer.[8,9] However, both S- and R-warfarin are also metabolized by CYP3A4.[8–10] We predicted AUCRs of 0.75 and 0.92 for induction of CYP2C9 from 3D spheroid PHHs based on mRNA and protein induction (Table 1). The AUCR based on mRNA induction in 2D cultured PHHs was similar (0.80, Table S2) to 3D spheroid PHHs (0.75, Table 1). In our previous clinical study with dicloxacillin,[7] the AUCR for tolbutamide was 0.73 indicating weaker clinical induction of CYP2C9 than CYP3A4, which aligns with the in vitro data here. Since the induction of CYP enzymes will affect also minor metabolic pathways for both enantiomers of warfarin, it is likely that the DDI between dicloxacillin and warfarin is a result of induction of both CYP2C9 and CYP3A4.

Direct oral anticoagulants (DOACs) are alternatives for warfarin and have partially replaced warfarin in clinical care. Regarding the DDI potential of dicloxacillin and flucloxacillin, DOACs are not an exception in comparison to warfarin. Most DOACs are substrates for the efflux transporter ABCB1 and partially metabolized by CYP3A4, and thus a combination of enzyme inducers and DOACs should be avoided.[43] Since both dicloxacillin and flucloxacillin also induce ABCB1,[25] close monitoring is needed when these antibiotics are administered to patients under a treatment with DOACs.

The registry-based analysis here was based on data on filled prescriptions, and thus the intake of prescribed dose for the prescribed duration cannot fully be ensured. Furthermore, in our analysis we cannot control for variation in INR levels caused by warfarin dose adjustments after dicloxacillin or flucloxacillin prescriptions. However, our analysis was based on large population and included 2 other antibiotics that did not decrease INR values and had comparable or higher number of individuals in the analysis. Although the in vitro data showed clear induction of CYPs by dicloxacillin, IVIVE is based on static model assuming metabolism of a victim drug by a single enzyme, which may lead to conservative estimates of DDIs. By contrast, we did not include intestinal induction in our predictions, and this exclusion may give lower estimates of magnitude of DDIs for drugs with extensive first-pass metabolism.

In conclusion, dicloxacillin decreases the efficacy of warfarin in a real-life setting and we show a substantially exacerbated effect during long-term treatment, which have major clinical impact for patients treated for endocarditis or other indications for long-term antibiotic treatments during warfarin therapy. This effect is attributed to induction of several clinically relevant CYP enzymes by dicloxacillin. Our in vitro predictions based on 3D spheroid PHHs indicate that dicloxacillin is a weak inducer of CYP2Cs, 2B6 and CYP3A4, which corresponds to clinical and observational studies. Caution regarding DDIs is warranted when patients are treated with dicloxacillin, during short- and especially long-term treatment particularly for drugs with narrow therapeutic index.

AUTHOR CONTRIBUTIONS

Erkka Järvinen, Ann-Cathrine Dalgård Dunvald and Tore B. Stage wrote the manuscript; Erkka Järvinen performed hepatocyte experiments and analysed in vitro data, Helen S. Hammer and Oliver Pötz performed proteomics experiments, Ann-Cathrine Dalgård Dunvald and Martin Thomsen Ernst analysed register-based data, Tore B. Stage and Anton Pottegård conceptualized the research, all authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

T.B.S. has given paid lectures for Pfizer and Eisai, consulted for Pfizer and collaborated with Novo Nordisk A/S. A.C.D. has given paid lectures for Astellas Pharma. All the above is unrelated to the work reported in this paper. A.P. reports participation in research projects funded by Alcon, Almirall, Astellas, Astra-Zeneca, Boehringer-Ingelheim, Novo Nordisk, Servier and LEO Pharma, all regulator-mandated phase IV-studies, all with funds paid to the institution where he was employed (no personal fees) and with no relation to the work reported in this paper. O.P. is a shareholder of SIGNATOPE GmbH. SIGNATOPE offers assay development and service using immunoaffinity-LC–MS/MS technology. All other authors declared no competing interests for this work.

DATA AVAILABILITY STATEMENT

In vitro data are available as supporting information of this article. Individual-level data from register-based study are not publicly available.

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